ORIGINAL ARTICLE

Expression of a Hoechst 33342 Efflux Phenomenon and Common Characteristics of Pluripotent Stem Cells in a Side Population of Amniotic Fluid Cells

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SUMMARY

Objective: The aim of this study was to verify the existence of a side population (SP) of cells in second-trimester amniotic fluid.

Materials and Methods: Amniotic fluid samples ($n=35$) were obtained, and the number and size of viable amniotic fluid cells (AFCs) were analyzed. Small AFCs (SAFCs) and large AFCs (LAFCs) were isolated using a sterile 10-μm pore size strainer. Hoechst 33342 dye exclusion assay, flow cytometry analysis, reverse transcriptase polymerase chain reaction and immunocytochemistry were used to analyze the characteristics of SAFCs and LAFCs.

Results: The mean concentration of viable AFCs from 16 to 21 weeks of gestation was 0.3×10^5, 0.8×10^5, 1.1×10^5, 1.3×10^5, 1.0×10^5 and 1.0×10^5 cells/mL respectively. The mean percentage of SAFCs from 16 to 21 weeks of gestation was 27.3%, 40.5%, 49.7%, 60.2%, 41.0% and 58.2%, respectively. The Hoechst 33342 efflux phenomenon was obvious among SAFCs but was rare in the LAFC population. Flow cytometry analyses showed that cell surface antigen expression on LAFCs and SAFCs were positive for CD29, CD44, CD73, CD90, CD166 and HLA-I, but negative for CD31, CD34, CD45, CD117 and HLA-II. Importantly, Nanog, Oct-4, ABCG2 and SOX2 expression in cells was easily detectable among the SAFC population. Expression of Nanog and ABCG2 was not observed among LAFCs.

Conclusion: Amniotic fluid contains a SP that was found mostly among the SAFCs. Enriched SP cells isolated by the efflux of Hoechst 33342 could be a novel and promising source of pluripotent-like amniotic derived stem cells for cellular therapy in the near future. [*Taiwan J Obstet Gynecol* 2010;49(2):139–144]

Key Words: amniotic fluid cells, amniotic fluid-derived stem cells, side population

Introduction

Amniotic fluid contains a heterogeneous population of cells of fetal origin. Potential sources contributing to the presence of cells in amniotic fluid are the fetal skin, the fetal membranes of placenta, and the epithelial and mucosa of the fetal digestive, respiratory and urinary tracts [1–3]. We previously developed a two-stage culture protocol to isolate a population of amniotic fluid-derived mesenchymal stem cells (AFMSCs) from second-trimester amniocentesis [4]. We further reported that human amniotic fluid contains a subpopulation of clonally multipotent fetal stem cells. Besides the common mesenchymal lineages, they also have the capacity to differentiate into multiple neural lineages and release dopamine *in vitro*. These findings supported the idea that human amniotic fluid provides a great

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alternative source of multipotent stem cells for the treatment of neurodegenerative disorders and central nervous system injuries [5].

The side population (SP) is a particular cell population enriched in primitive and undifferentiated stem cells. The isolation and enrichment of a SP fraction is based on the efflux of the fluorescent dye, Hoechst 33342, as reported by Goodell et al [6] in 1996. Most researchers consider that the SP is an enriched source of stem cells for cellular therapy. Although SP cells have been isolated from various adult tissues such as hematopoietic bone marrow, skeletal muscle, brain, liver, pancreas, lung, heart and kidney [7–9], there have been no published reports of isolated SP cells from human amniotic fluid to date.

In this study, we report a population of small amniotic fluid cells (SAFCs) that had Hoechst 33342 efflux capacity and express common markers of pluripotent stem cells.

Materials and Methods

Cell size analysis of viable amniotic fluid cells

A total of 2–3 mL of amniotic fluid was obtained by routine amniocentesis at 16–20 weeks of gestation for fetal karyotype analysis. After centrifugation, cells were suspended in phosphate buffered saline (PBS) (Sigma, St. Louis, MO, USA) and the size of viable amniotic fluid cells (AFCs) was analyzed with a Cedex HiRes Cell Analyzer (Roche Innovatis, Bielefeld, Germany) following the manufacturer's instructions. The institutional review board of Cathay General Hospital approved this protocol (CGHIRB no. CT9558), and each patient gave written informed consent.

Isolation of a subpopulation of SAFCs

Dead epithelial cells were removed from the amniotic fluid with a 40-μm pore size strainer. The sterile 10-μm strainer (Millipore, Bedford, MA, USA) was used to isolate smaller cells from native AFCs. The cells that passed through the strainer were SAFCs, and those that were retained were resuspended and collected as large AFCs (LAFCs). Both the SAFCs and LAFCs were analyzed. AFCs were suspended in minimum essential medium alpha, (Hyclone, Logan, UT, USA) containing 20% (vol/vol) fetal bovine serum (FBS) (Hyclone), basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA) at 4 ng/mL, penicillin at 100 U/mL and streptomycin (Sigma) at 100 μg/mL, and were incubated at 37°C in a 5% humidified CO_2 atmosphere. The non-adherent cells in the supernatant medium were removed 3 days after seeding, and the culture medium was changed twice a week. When the cells reached 90% confluence, they were washed, harvested with 0.05% trypsin-EDTA (Sigma) and passaged at a ratio of 1:3 to 1:4.

Hoechst 33342 dye exclusion assay

Both the SAFCs and LAFCs were centrifuged at 200*g* for 10 minutes, and incubated in minimum essential medium alpha with 5% FBS and 5 μg/mL Hoechst (Sigma) at 37°C for 1 hour. After PBS washing, the positive stained cells were observed under fluorescence phase-contrast microscopy.

Flow cytometry analysis

For characterization of SAFCs, the cells were trypsinized and resuspended in PBS. After fixing with 1% methanol, the cells were blocked in 1% bovine serum albumin (Sigma) with 0.1% FBS for 1 hour at room temperature, then washed with three volumes of PBS. For direct analysis, cells were incubated with the following mouse anti-human antibodies: CD26-FITC, CD29-PE, CD31-FITC, CD34-PE, CD44-FITC, CD45-FITC, CD73-PE, CD90-FITC, CD117-PE, CD166-PE, HLA-ABC-PE and HLA-DR-PE (all from Becton-Dickinson, San Jose, CA, USA). The analysis was performed on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) with at least 30,000 events recorded.

Reverse transcriptase-polymerase chain reaction

The SAFCs were frozen at –80°C to release nucleic acids for analysis. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using OneStep assay kits (Qiagen, Santa Clarita, CA, USA), following the manufacturer's instructions, and the expression of β-actin was used for normalizing sample templates. The PCR amplification was performed over 35 cycles with specific primers for β-actin (forward, 5′-GCA CTC TTC CAG CCT TCC TTC C-3′; reverse, 5′-TCA CCT TCA CCG TTC CAG TTT TT-3′), Sox2 (forward, 5′-AGA ACC CCA AGA TGC ACA AC-3′; reverse, 5′-GGG CAG CGT GTA CTT ATC CT-3′), Oct-4 (forward, 5′-CGT GAA GCT GGA GAA GGA GAA GCT G-3′; reverse, 5′-CAA GGG CCG CAG CTT ACA CAT GTT C-3′), Nanog (forward, 5′-GCG CGG TCT TGG CTC ACT GC-3′; reverse, 5′-GCC TCC CAA TCC CAA ACA ATA CGA-3′); ABCG2 (forward, 5′-AGT TCC ATG GCA CTG GCC ATA-3′; reverse, 5′-TCA GGT AGG CAA TTG TGA GG-3′). The PCR products were subjected to electrophoresis on 1.3% (wt/vol) agarose gels and visualized by ethidium bromide staining.

Immunocytochemical staining

Fresh AFCs were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) and permeabilized with

0.1% Triton X-100 (Sigma). After blocking with 10% normal blocking serum (derived from the same species as the secondary antibody) in PBS for 30 minutes, cells were incubated with primary antibodies against ABCG2, Nanog, Oct-4 or Nestin (all from Chemicon, Temecula, CA, USA) for 1 hour. After having been washed twice with PBS, cells were incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. The resulting immunoreactive cells were visualized by fluorescence microscopy.

Results

In the initial experiments, we used a Cedex HiRes Cell Analyzer to assess the number of viable AFCs in native second-trimester amniotic fluid. Two subpopulations of viable AFCs were easily identified by their difference in size. The average diameter of the small and large cell populations was 5 μm (range, 3–8 μm) and 12 μm (range, 10–20 μm), respectively (Figure 1A). The subpopulation of viable SAFCs could be isolated using a 10-μm pore size strainer as shown in Figure 1B. The mean concentration of viable AFCs was 0.3×10^5 cells/mL at 16 weeks, 0.8×10^5 cells/mL at 17 weeks, 1.1×10^5 cells/mL at 18 weeks, 1.3×10^5 cells/mL at 19 weeks, 1.0×10^5 cells/mL at 20 weeks, and 1.0×10^5 cells/mL at 21 weeks (Figure 2A). The mean percentage of SAFCs was 27.3% at 16 weeks, 40.5% at 17 weeks, 49.7% at 18 weeks, 60.2% at 19 weeks, 41.0% at 20 weeks, and 58.2% at 21 weeks (Figure 2B).

We observed a subpopulation of SAFCs that had the capacity to exclude Hoechst 33342, thereby demonstrating the existence of a SP among SAFCs derived from fresh second-trimester amniotic fluid (Figure 3A). Hoechst 33342 efflux in LAFCs was rare (Figure 3B). The Table outlines the specific surface antigenic characteristics of the cultured SAFCs and LAFCs at passage 4 as determined by flow cytometry. The analyses showed that the cells of these two populations were positive for CD29, CD44, CD73, CD90, CD166 and HLA-I, but negative for CD31, CD34, CD45, CD117 and HLA-II. The difference between these two cell types was in the expression of CD26, where LAFCs strongly expressed this molecule after culturing but SAFCs were only weakly positive. Further characterization studies were performed using RT-PCR and immunocytochemical staining for the detection of Nestin, a marker of neural stem cells, and ABCG2, Nanog, Oct-4 and Sox2, markers of pluripotent stem cells. LAFCs expressed high levels of Sox2 and low levels of Oct-4, whereas SAFCs were positive for Nanog, Oct-4 and SOX2 by RT-PCR (Figure 4). However, only SAFCs expressed Nestin, ABCG2 and

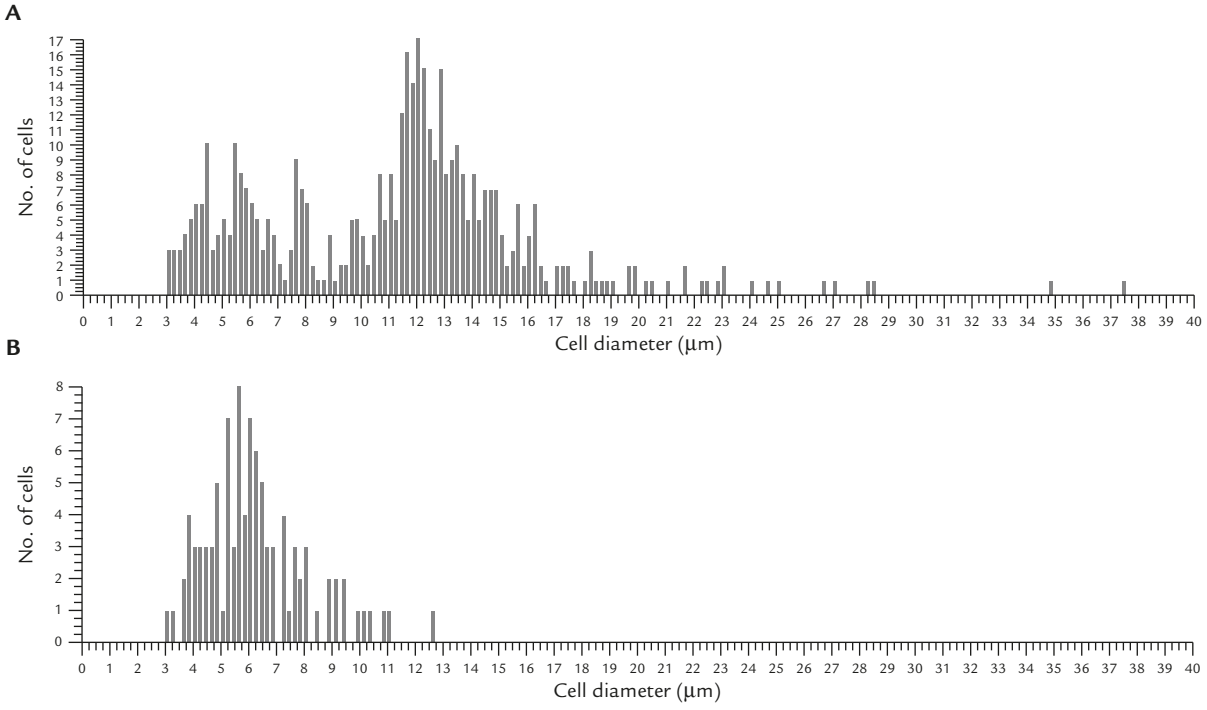

Figure 1. Viable amniotic fluid cells (AFCs) were analyzed with a Cedex HiRes Cell Analyzer (Roche Innovatis, Bielefeld, Germany). (A) Two populations of AFCs were identified. The median cellular diameters of small and large AFCs were 5 μm and 13 μm, respectively. (B) The small AFC population was isolated using a 10-μm strainer.

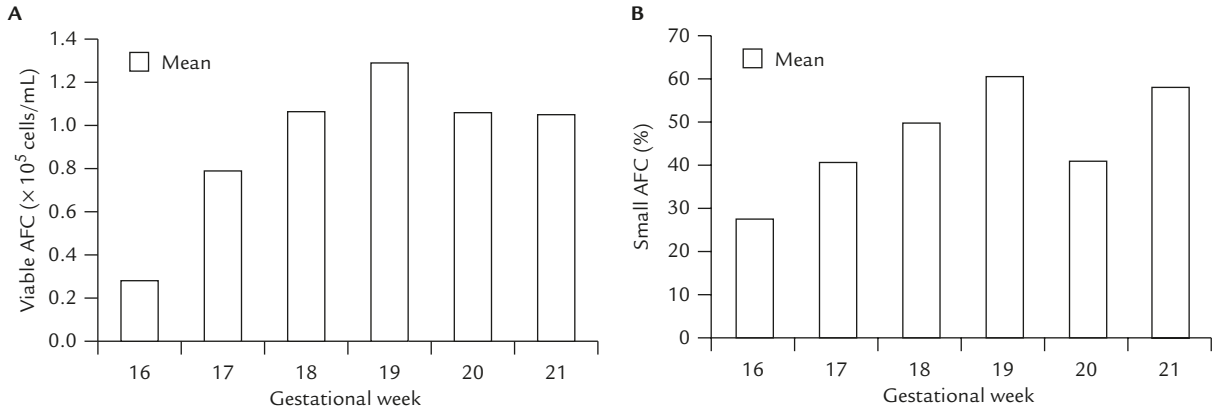

Figure 2. The numbers of viable amniotic fluid cells (AFCs) from 16 to 21 weeks of gestation. (A) The mean numbers of viable AFCs at each week. (B) The mean percentage of small AFCs at each week.

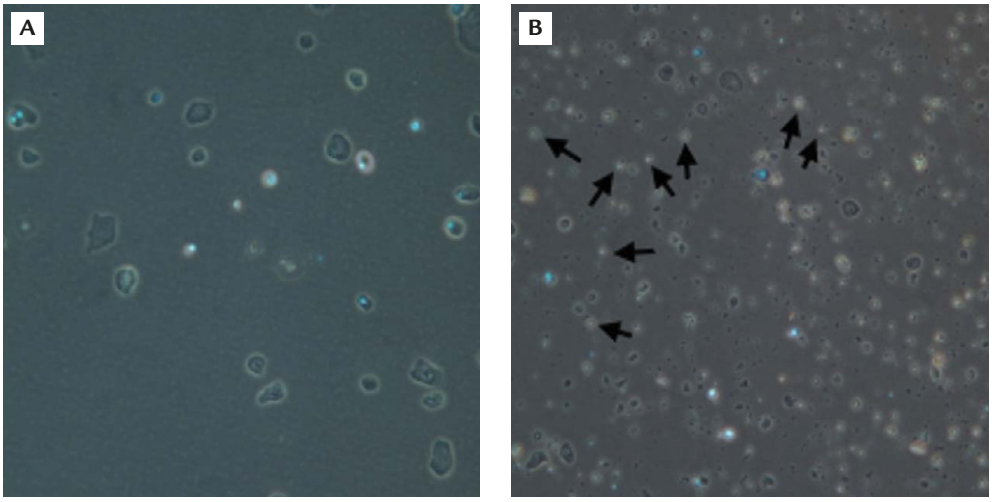

Figure 3. Overlay of brightfield and ultraviolet images displaying the positive Hoechst 33342 efflux phenomenon among the small amniotic fluid cells (AFCs). (A) Hoechst 33342 exclusion was not noted in large AFCs. (B) The arrows indicate Hoechst 33342 excluding cells in small AFCs.

Nanog, as determined by immunocytochemical staining (Figure 5).

Discussion

Prusa et al [10] were the first to report evidence of human amniotic fluid containing stem cells in 2003. They identified Oct-4-expressing cells in human amniotic fluid and proposed that these might be a new source for pluripotent-like stem cells with the added benefit that their use might avoid the ethical issues associated with the use of human embryonic stem cells [10]. In the same year, in 't Anker et al [11] demonstrated that human amniotic fluid was an abundant source of fetal mesenchymal stem cells (MSCs), and these cells exhibited a phenotype and multilineage differentiation potential similar to that of bone marrow-derived MSCs. They suggested that these amniotic fluid-derived MSCs could be used for cotransplantation in conjunction with umbilical cord blood-derived hematopoietic stem cells [11]. In 2004, Tsai et al [4] successfully obtained AFMSCs using a novel two-stage culture protocol without interfering with the process of fetal karyotyping. In that study, the authors were able to demonstrate that AFMSCs could be expanded rapidly, and maintained the capacity to differentiate into multiple cell types such as adipocytes, osteoblasts and neurons *in vitro* [4]. In addition to constituting a breakthrough in obtaining human fetal stem cells from non-adherent cells of a backup culture, this valuable discovery also offers a solution to the ethical dilemmas associated with obtaining stem cells from human embryos and fetuses.

In the present preliminary study, we further demonstrated that second-trimester amniotic fluid contains two subpopulations of viable AFCs, the LAFCs and SAFCs, which can be easily separated courtesy of the difference in their cellular diameter. Both the LAFC and

Table. Phenotypic characteristics of small and large amniotic fluid cells (AFCs)

	Small AFC (P4)	Large AFC (P4)
CD26	+/−	+
CD29	+	+
CD31	−	−
CD34	−	−
CD44	+	+
CD45	−	−
CD73	+	+
CD90	+	+
CD117	−	−
CD166	+	+
HLA-I	−	−
HLA-II	+	+

P4 = passage 4.

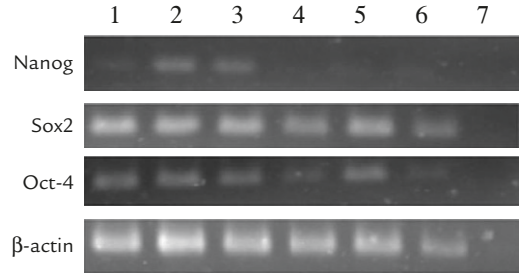

Figure 4. Reverse transcriptase-polymerase chain reaction analyses of the common embryonic stem cell markers in large amniotic fluid cells (AFCs) and small AFCs. Small AFCs expressed Nanog, Oct-4, and SOX2 mRNA at high levels (lanes 1–3: three individual small AFCs); large AFCs expressed Sox 2 at a high level and Oct-4 mRNA at a low level, and did not express Nanog mRNA (lanes 4–6: three individual large AFCs). Lane 7: negative control.

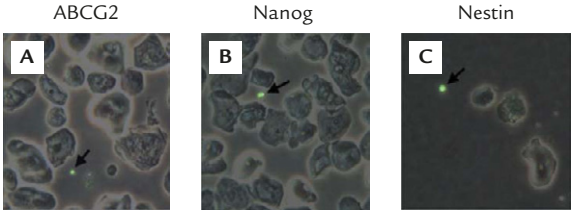

Figure 5. Immunocytochemical analyses reveal that small amniotic fluid cells populations contain (A) ABCG2- (arrow), (B) Nanog- (arrow) and (C) Nestin-expressing cells (arrow).

SAFC populations have the same phenotypic characteristics, and these are similar to MSCs derived from other sources such as first-trimester fetal tissues [12,13], term umbilical cord blood [14,15], and placenta [16,17] which are positive for CD29, CD44, CD73, CD90, CD166 and HLA-I, but negative for CD31, CD34, CD45, CD117 and HLA-II. The only difference in cell surface antigen expression between these two populations involved CD26. It was present at a high levels on the surface of LAFCs, but maintained at low levels on SAFCs after culturing. However, both LAFCs and SAFCs can be expanded rapidly and maintain the capacity to differentiate into multiple cell types *in vitro*, which is the same as for AFMSCs as described in previous publications [4,5]. Importantly, SAFCs contain a subpopulation of pluripotent-like stem cells that expressed high levels of Nanog, a gatekeeper of pluripotency in human embryonic stem and carcinoma cells which prevents their differentiation into extraembryonic endoderm and trophectoderm lineages, and Oct-4, a transcription factor expressed in embryonic stem cells, reflecting a key role in the maintenance of stem cell pluripotency *in vivo* and *in vitro* [5,10]. Only a few Oct-4-expressing cells were found amongst the LAFCs. Whether LAFCs and SAFCs are stem cells derived from different fetal tissues or from the same fetal origin at different stages of cell maturation is an issue worth further investigation.

The ATP-binding cassette (ABC) transporters represent a family of proteins with the capacity to bind ATP as an energy source to transport endogenous or exogenous molecules across the cellular membrane. ABCG2, a member of the ABC transporter protein family and also a molecular determinant of SP phenotype, has been identified in the placenta, brain, kidney, intestine, liver, testis, and ovaries. There is increasing evidence showing that ABC transporters repress the maturation and differentiation of stem cells [18–21].

In addition to the ability to exclude Hoechst 33342 dye, SAFCs also expressed ABCG2, suggesting that SAFCs may contain a subpopulation of primitive and undifferentiated stem cells with pluripotent potential. This original discovery indicates that SP cells truly exist in the native human amniotic fluid and are mostly found among the SAFC population, which is consistent with the notions that SP cells are smaller than non-SP cells as described by Benchaouir et al [22], and that small cell size is a stem cell characteristic as acknowledged by Paiva et al [23].

Based on these findings, utilizing Hoechst 33342 dye efflux activity to isolate SP cells from amniotic fluid by flow cytometry could become a novel and promising strategy for isolating enriched amniotic fluid-derived stem cells, both for cellular therapy and for stem cell engineering in the future.

In 2007, De Coppi et al [24] isolated the c-kit (CD117) positive population by immunoselection from second-trimester amniotic fluid samples and demonstrated that amniotic fluid-derived stem cells have pluripotent potential like embryonic stem cells, and are able to differentiate into adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic lineages. Whether the SP cells of amniotic fluid have the same

pluripotent potential and are abundant enough for cellular therapy is an interesting issue that requires further exploration and is under investigation.

Acknowledgments

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